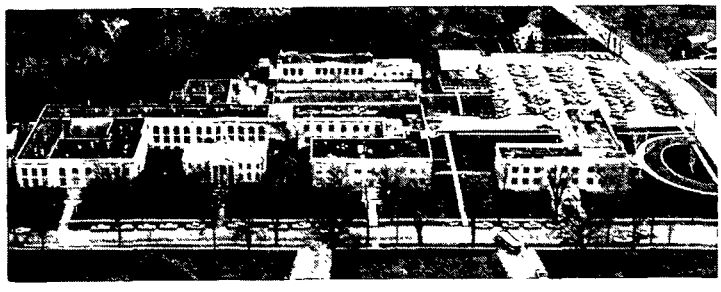

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES
NUMBER 93

METHYLGLYOXAL: METABOLISM IN DOUGLAS–FIR NEEDLES AND DOUGLAS–FIR CALLUS

METHYLGLYOXAL: PRESENCE IN DOUGLAS–FIR NEEDLES AND ABSENCE IN DOUGLAS–FIR NEEDLE CALLUS

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MARCH, 1980

METHYLGLYOXAL: METABOLISM IN DOUGLAS-FIR NEEDLES AND DOUGLAS-FIR NEEDLE CALLUS

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SUMMARY

Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] needle callus was found to contain a strong complement of enzyme systems capable of catabolizing methylglyoxal. Methylglyoxal synthetase, the enzyme which converts dihydroxyacetone phosphate to methylglyoxal, could not be detected in extracts of needle callus. In extracts of Douglas-fir needles, glyoxalase I activity was observed only after isoelectric focusing. A study of needles at various stages of development revealed a potentially significant relationship between methylglyoxal reductase and synthetase activities. Normal development in Douglas-fir needles appears to involve a modulation of these two enzymes, resulting in the control of endogenous methylglyoxal levels.

These papers have been submitted for publication in Plant Science Letters.

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INTRODUCTION

The results in the accompanying paper [1] provided evidence that methylglyoxal may be acting as a natural cell division inhibitor in plants as envisioned for aerobic organisms by Szent-Gyorgyi [2-5]. Yet to be answered, however, is whether or not this finding is consistent with the distribution of enzymes controlling the metabolism of methylglyoxal. According to Szent-Gyorgyi, the glyoxalase enzymes must be kept separated from the methylglyoxal in order to maintain the resting state. Since the mature needles, which were found to contain methylglyoxal, are viewed as representatives of the resting state, they should contain bound (inactive) glyoxalase. The needle callus, which lacks methylglyoxal and is viewed as representative of the dividing state, should contain free glyoxalase. By analogy with animal systems, it could be postulated that the wounding of needles (initiating callus formation) causes the release of bound glyoxalase with the resultant catabolism of methylglyoxal as central to the transition to the proliferative needle callus state.

To examine more thoroughly the metabolism of methylglyoxal requires more than just consideration of the glyoxalase system. Such a study should include an analysis for all known enzymatic pathways involved in the metabolism of the compound. The mechanism of *in vivo* formation of methylglyoxal had long been a mystery. However, in 1970 Cooper and Anderson reported an enzyme from *Escherichia coli* which catalyzed the formation of methylglyoxal from dihydroxyacetone phosphate [6]. The enzyme was named methylglyoxal synthetase and has since been isolated from *Proteus vulgaris* as well [7].

The pathway usually considered for the degradation of methylglyoxal is through D-lactate by the glyoxalase system. The generally accepted reaction mechanism involves glyoxalase I converting the chemical addition product between methyl-

glyoxal and reduced glutathione to the thioester. The thioester is then hydrolyzed by glyoxalase II generating D-lactate and releasing reduced glutathione [8]. More recently, two other enzyme systems have been shown to exist which degrade methylglyoxal. In 1967 Monder reported an enzyme from sheep liver which catalyzed the oxidation of methylglyoxal directly to pyruvate using NAD^+ or $NADP^+$ [9]. The enzyme, α-ketoaldehyde dehydrogenase, has since been reported to exist in Bacillus subtilis [10] and rat liver [11]. The third route for the catabolism of methylglyoxal is via lactaldehyde due to the action of methylglyoxal reductase [10]. This enzyme requires either NADH or NADPH and has been reported in Bacillus subtilis.

METHODS AND MATERIALS

Tissue Samples Douglas-fir needles were obtained from seedlings grown under greenhouse conditions. Douglas-fir needle callus was initiated from seedling needles and grown on a modified Murashige and Skoog medium containing 5 ppm napthaleneacetic acid and 0.1 ppm of N^6-benzylaminopurine. Fluorescent lamps provided the lighting at from 125-175 footcandles with a constant day length at 23°C. Tissue was subcultured onto fresh medium once every month.

Sample Preparation All tissues were prepared for enzyme assays by making acetone powders. The callus tissue was homogenized in a Ten Broeck grinder. The acetone as well as the homogenizer and centrifuge tubes were kept at -18°C. The tissues were homogenized in the cold acetone (5:1, v/w), and the suspension was centrifuged in a Sorvall RC2-B centrifuge at 27,000 x g for 15 min at -15°C. The pellet was then extracted with buffer, giving an aqueous enzyme solution. The needles were first subjected to cold acetone in a Virtis homogenizer at 0.5 amps for one min. This suspension was centrifuged as before

and the pellet extracted a second time with cold acetone using the Ten Broeck. Following a second centrifugation, the resultant pellet was extracted with buffer for its enzyme complement.

The buffer extraction procedure involved adding 2.0 cc of cold 50 mM potassium phosphate buffer at pH 6.8 (or Tris at pH 7.4 if glyoxalase II was to be assayed) per g fresh wt. of tissue to the pellet in the centrifuge tube. The contents were stirred continuously in the cold at 2-3°C for one h. Following centrifugation the supernatant was decanted and used directly in the enzyme assays.

Enzyme Units All enzyme units are expressed as nanomoles of product produced or substrate decomposed per min. at 25°C. The molar absorptivity coefficient of S-lactoylglutathione used for the glyoxalase enzyme system was 3370^{-1} cm^{-1} at 240 nm [8]. Those enzymes requiring either of the two nicotinamide-containing coenzymes were assayed at 340 nm using 6220^{-1} cm^{-1} as the molar absorptivity coefficient. The rate of reaction was determined from the slope of the initial two-minute linear portion of the plot.

Enzyme Assays A Perkin-Elmer 576 recording spectrophotometer was used for all enzyme activity measurements in 1.0 cm quartz cuvettes at 25°C. All solutions were prepared with double-distilled water. No activity was observed in the absence of added enzyme or substrate.

Glyoxalases I and II were assayed by modifications of the method of Racker [8]. The glyoxalase I reaction was measured by following the increase in absorbance at 240 nm due to the formation of the thioester. The sample contained 3.3 µmoles of reduced glutathione, 17.8 µmoles of methylglyoxal, 30.0 µmoles of $MgCl_2$, 125.0 µmoles of potassium phosphate buffer at pH 6.8, and 0.10 cc of

enzyme extract in a total volume of 3.0 cc. For glyoxalase II, the decrease in absorbance of the thioester at 240 nm was followed. The sample cuvette contained 1210 μmoles of S-lactoylglutathione, 137.5 μmoles of Tris at pH 7.4, and 0.20 cc of enzyme extract in a total volume of 3.0 cc. In both assays the reference cuvette contained the same constituents except for the enzyme extract in a total volume of 3.0 cc. Methylglyoxal, glutathione, and S-lactoylglutathione were purchased from Sigma Chemical Co.

Methylglyoxal reductase was measured by the method of Willetts and Turner [10] by recording absorbance changes at 340 nm. The coenzymes NADH and NADPH were purchased from P-L Biochemicals. The oxidation of methylglyoxal by α-ketoaldehyde dehydrogenase was determined by the method of Monder [9] at 340 nm. The coenzymes NAD^+ and $NADP^+$ were products of P-L Biochemicals. The glyoxalase I-coupled reaction of Hopper and Cooper [12] was used to assay methylglyoxal synthetase activity. Glyoxalase I and dihydroxyacetone phosphate were purchased from Sigma.

Isoelectric Focusing Isoelectric focusing was carried out on prefocused Ampholine PAGplate gels with a pH gradient from 3.5 to 9.5. The anode solution consisted of 1 M H_3PO_4 and the cathode solution was 1 M NaOH. Constant power of 25 w was applied for two h. Upon completion of the focusing period, the gel was stained either for protein or selectively for enzyme activity. Glyoxalase I activity was determined on the gel using the specific stain MTT tetrazolium according to Aronsson and Mannervik [13]. The MTT tetrazolium was purchased from Sigma. The isoelectric focusing gels and apparatus were products of LKB.

RESULTS

The analysis of several clones of Douglas-fir needle callus for glyoxalase I and II activity is represented here by two different clones signified as 15

and 94, which had been in culture 10 months and 26 months respectively. The enzyme assays were conducted once a week over a period of eight consecutive weeks. Both clones exhibited glyoxalase I and II activity as is shown in Table I. Depending on the time since subculturing as well as the clone, the glyoxalase I activity in needle callus ranged from 400-1400 units per g fresh wt and glyoxalase II, from 100-650 units per g fresh wt. Analysis for α-keto-aldehyde dehydrogenase in these two clones was negative. Both clones contained methylglyoxal reductase activity which was found to range from 200 to 300 units per g fresh wt. Douglas-fir needle callus contained two of the three possible methylglyoxal catabolic enzymes. No methylglyoxal synthetase activity could be elicited from any of the needle callus preparations.

[Table I here]

Neither glyoxalase I nor II activity could be detected in crude preparations from needles. In fact, as little as 50 microliters of needle extract, representing 1/100th of the total extract from 2.97 g of needles, totally inhibited 17 units of needle callus glyoxalase I. The same volume of extract inhibited 1,029 units of commercial enzyme by 92%. Since the needle extract was strongly inhibitory to glyoxalase I, it was isoelectrically focused in hopes of removing the inhibitor. Direct analysis of the gel with MTT as well as spectrophotometric assay of a buffer extract of the gel after focusing confirmed the presence of glyoxalase I. The glyoxalase I activity in needles was quantitated at 45 units per g fresh wt. No α-ketoaldehyde dehydrogenase activity was detected in the crude needle preparations.

Since glyoxalase activity in mature needle extracts was found to be strongly inhibited, it was considered that young developing needles might contain active glyoxalase. To test this idea, we collected needles from 64-week old seedlings

and divided them into three categories. One group consisted of early flush material 1.0-1.5 cm long, another of fully elongated light green needles 2.5-3.0 cm long, and the third group consisted of mature dark green needles 2.5-3.0 cm in length. Again, no glyoxalase activity could be elicited from any sample. Methylglyoxal reductase activity, however, was present and decreased in a linear fashion with increasing age of the needles. On the other hand, methylglyoxal synthetase activity was high in the 2.5-3.0 cm fully elongated flush material with no activity in the other needle samples. A summary of enzyme activities in the various tissue types is given in Table II.

[Table II here]

DISCUSSION

The accompanying paper [1] suggested that methylglyoxal, which was isolated from Douglas-fir needles, might be acting as a cell division inhibitor in these plants as envisioned by Szent-Gyorgyi. Reported in the same study was that a similar preparation from Douglas-fir needle callus contained no methylglyoxal, which might explain the observed proliferation of callus. The results reported here show that these findings are consistent with the distribution of methylglyoxal-related enzymes.

An early indication that needle callus cells might contain a strong complement of methylglyoxal-degrading enzymes was shown with suspension cells. Work in this laboratory [14] had demonstrated that 0.1 mM methylglyoxal inhibited the growth of needle suspension cells by 50% after one month of culture, based upon dry weights. However, the cells recovered from this inhibition, indicating that the methylglyoxal was being degraded. Additional research demonstrated the presence of glutathione as the major component of the nonprotein sulfhydryl fraction in needle callus. This assured us that if glyoxalase was present, so was the necessary cofactor.

Douglas-fir needles contain methylglyoxal and the enzyme to synthesize it. Glyoxalase I activity was found in the needles only after isoelectric focusing, indicating strong inhibition; it is not known whether the inhibitor is functional in vivo. Conceivably, in the young needles cell division is protected from the inhibitory action of methylglyoxal by methylglyoxal reductase. As the needle develops and reaches its fully elongated stage, methylglyoxal reductase activity decreases and methylglyoxal synthetase activity peaks. This might allow the buildup of methylglyoxal which could act as a "brake" on futher cell division. After the needle matures, neither of the enzyme activities can be found, and the cells remain in a resting state. The proper modulation of methylglyoxal reductase and synthetase activities along with inhibition of glyoxalase could play an important role in normal needle development.

The proliferative Douglas-fir needle callus cells contain no methylglyoxal nor the enzyme to synthesize it. Their cell division is completely unchecked by the possible natural "brakelike" action of methylglyoxal. Furthermore, any methylglyoxal entering the needle callus cells would be rapidly metabolized because of the presence of both glyoxalase and methylglyoxal reductase, explaining the need for rather high concentrations to reversibly inhibit suspension cells as noted. The reversibility of methylglyoxal inhibition, which is essential to the proposed role for this compound, was also observed in 1974 by Mankikar and Rangekar in their study of barley seed germination [15]. Since needle callus tissue is the result of a wounding action, the presence of glyoxalase in needle callus could be viewed as a release from the inhibition found in needles. In general, the distribution of methylglyoxal and enzyme activities concerned with its metabolism observed in this study tend to support the cell division hypothesis of Szent-Gyorgyi.

Some limited data we obtained from tobacco, tobacco crown gall and tobacco callus [14] suggests that this mechanism might be widespread and might well underlie the cancer problem as Szent-Gyorgyi claims. Ironically, conifers were thought to be a possible exception to the universal distribution of glyoxalase in a study conducted with indirect methodology in 1945 [16].

ACKNOWLEDGEMENT

We thank Dr. L.L. Winton and Mrs. Shirley Verhagen for their assistance in tissue culture aspects of this research which was supported by the pulp and paper industry through The Institute of Paper Chemistry. Portions of this work were used by one of the authors (M.M.S.) as partial fulfillment of the requirements for the Ph.D. degree at The Institute of Paper Chemistry.

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TABLE I

GLYOXALASE I AND II ACTIVITY IN DOUGLAS-FIR NEEDLE CALLUS CLONES 15 AND 94 RELATIVE TO SUBCULTURE TIME

(The results are given as units per gram fresh weight and are the means of three determinations)

Sample	Time Since Subculturing, weeks[a]							
	1	2	3	4	1	2	3	4
Clone 15								
Glyoxalase I	848	433	600	555	537	717	1045	1366
Glyoxalase II	244	138	231	94	268	326	498	334
Clone 94								
Glyoxalase I	1079	666	940	511	688	1055	1194	1250
Glyoxalase II	324	296	486	400	364	657	644	652

[a]Tissue subcultured after week 4.

TABLE II

SUMMARY OF ENZYME ACTIVITIES IN CRUDE PREPARATIONS OF DOUGLAS-FIR NEEDLE AND NEEDLE CALLUS TISSUES

(The results are given as units per gram fresh weight and are the means of three determinations)

Sample	Glyoxalase I	Glyoxalase II	Methylglyoxal Reductase	Methylglyoxal Synthetase
1.0-1.5 cm needles	0	0	58	0
2.5-3.0 cm needles	0	0	29	97
2.5-3.0 cm mature needles	0	0	0	0
Clone 15	1250	652	200	0
Clone 94	1045	498	226	0

METHYLGLYOXAL: PRESENCE IN DOUGLAS-FIR NEEDLES AND ABSENCE IN DOUGLAS-FIR NEEDLE CALLUS

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SUMMARY

Methylglyoxal was isolated as its 2,4-dinitrophenylosazone from an insoluble fraction from Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] needles but was not observed in a similar Douglas-fir needle callus preparation. This finding lends considerable support to the idea that methylglyoxal plays a significant role in the control of cell proliferation. Its presence in needles could allow it to assume an inhibitory or "brakelike" action on cell division whereas its absence in needle callus could allow the observed proliferation. The data indicate that methylglyoxal may be operating in conifers as a cell division inhibitor as envisioned by Szent-Gyorgyi.

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INTRODUCTION

Albert Szent-Gyorgyi has proposed [1-9] that methylglyoxal keeps cells in the resting state from which they can be released by the glyoxalase enzymes for which glutathione is a cofactor. This investigation set out to examine that idea by using Douglas-fir needle tissue and Douglas-fir needle callus as representatives of resting (organized) and proliferative (unorganized) states, respectively. Since the needles are viewed as representative of the resting state in this study, they should contain methylglyoxal whereas the needle callus should not.

According to the hypothesis, methylglyoxal performs its function by reacting with sulfhydryl groups on enzymes controlling cell division as well as by complexing to the structural protein of membranes through charge-transfer reactions [9, 10, 11]. By masking the vital sulfhydryl groups of these enzymes they become nonfunctioning. Meanwhile, the charge-transfer reaction between methylglyoxal and structural protein is thought to add rigidity to the membranes.

In attempting to isolate methylglyoxal from the tissue sources, we could have examined two possible bound sites in each, soluble globular proteins or insoluble structural proteins. A preparation containing structural protein was chosen because of a recent successful isolation of methylglyoxal from beef liver structural protein by Fodor et al. [12]. In addition, an insoluble fraction would be relatively free of other soluble carbonyl compounds.

METHODS AND MATERIALS

The isolation and identification of methylglyoxal from Douglas-fir needles was by means of its 2,4-dinitrophenylosazone. The technique employed was a

slight modification of the procedure of Fodor et al. [12]. Mature needles (900 g fresh wt.) were collected from 56-week old Douglas-fir seedlings grown under greenhouse conditions. The Douglas-fir needle callus sample consisted of 407 g fresh wt. of callus cells. Culturing has been described previously [13]. Commercial methylglyoxal was obtained from Sigma Chemical Co.

Sample Preparation The needles and needle callus were homogenized three times in cold acetone at -18°C and vacuum filtered on a Buchner funnel with Whatman No. 1 filter paper. The resulting white powders were spread out on a tray and allowed to air dry overnight to remove residual acetone. The powders were then homogenized in double-distilled water, the pH adjusted to 4.5, and again homogenized. Each of the resulting suspensions was centrifuged for ten min at 10,000 x g. The supernatants were discarded.

Derivatization with 2,4-Dinitrophenylhydrazine The residues were suspended in 1 N HCl, the needles, in 1.0 liter and callus, in 500 mL. Under strong stirring, 5.0 g of 2,4-dinitrophenylhydrazine were added to the needle sample and 2.5 g, to the callus sample. Each of the mixtures was stirred for six days at room temperature and was then homogenized in ethyl acetate. The suspensions were centrifuged for 15 min at 10,000 x g. The ethyl acetate fractions were washed with double-distilled water and dried over anhydrous Na_2SO_4. After gravity filtration the ethyl acetate was removed with a rotary evaporator. The remaining residues were washed eight times with 50 mL portions of 60-90°C petroleum ether and then extracted six times with 50 mL portions of benzene. The benzene extracts were gravity filtered, and the benzene was removed with a rotary evaporator. The final residues were taken up in chloroform in preparation for chromatography.

Chromatographic Method The chloroform solutions were chromatographed on 1,000-micron thick Silicagel GF plates with toluene:60-90°C petroleum ether: ethyl acetate (34:5:7). The colored band corresponding in R_f to known methylglyoxal osazone was removed from the plates and eluted from the Silicagel with chloroform. This material was chromatographed in benzene:chloroform (2:1), and the corresponding bands were again removed and rechromatographed a final time in the original solvent. Bands at the proper R_f values were then removed and characterized.

Characterization The UV-VIS spectrum in chloroform, the VIS spectrum in 10% KOH in 80% methanol, the IR spectrum in Nujol, and the melting point were determined. All UV-VIS spectra were taken on a Perkin-Elmer model 576 recording spectrophotometer. The melting point was run on a Thomas Hoover capillary apparatus.

RESULTS

After derivatization and extraction with ethyl acetate, the residue from the benzene extract was dissolved in chloroform for both the Douglas-fir needle and needle callus samples. This chloroform fraction, still containing a number of derivatized carbonyl compounds in the needle sample, was chromatographed on Silicagel GF plates. For the needle sample the chromatogram showed a spot of considerable size corresponding in R_f to that of the osazone made from commercial methylglyoxal. No spot existed for the needle callus sample at the same R_f value. Both samples had spots corresponding to the reagent itself and the acetone derivative. The osazone area of the needle sample was removed and rechromatographed in two additional solvent systems. The clean single band corresponding in R_f to that of the known methylglyoxal osazone was then removed and characterized.

The isolate was quantified by the method of Wells [14]. The value obtained was 4.5×10^{-9} moles of methylglyoxal per g fresh wt. of needles. This was followed by an IR spectrum in Nujol which is compared to that published for the known methylglyoxal osazone [12] in Fig. 1. Additional characterization of the isolate was obtained by comparing spectra to that of the known derivative in methanolic KOH and chloroform (Fig. 2 and 3). Again, good spectral matches were obtained. Finally, the melting point was determined and found to be 303°C uncorrected as compared to a reported melting point of 309°C [15].

[Fig. 1-3 here]

To examine the possible contribution of artifact by certain carbohydrate and lignin moieties in the isolation procedure, we brought several additional substances through the same process. Thus, samples of dioxane lignin, cellulose, xylan, and arabinogalactan were treated with 2,4-dinitrophenylhydrazine in the same manner as the needle and needle callus samples. Spectral studies of these samples showed no interference. It is thus very likely that the methylglyoxal found in the needles was complexed to insoluble proteins via a charge-transfer reaction.

DISCUSSION

The existence of methylglyoxal in living systems has long been an elusive subject. While many studies claimed that methylglyoxal was formed by glycolyzing tissues [15, 16, 17], its formation was later considered antifactual, and its role in glycolysis was dismissed by Meyerhof [18]. Meyerhof and Lohmann [19] had demonstrated the conversion of triose phosphates to methylglyoxal in a nonenzymatic reaction catalyzed by acid. The published reports of methylglyoxal

formation in glycolyzing tissues were thus thought to occur by these nonenzymatic side reactions.

The discovery of methylglyoxal in a retine extract from thymus gland by Egyud in 1965 [20] caused a renewed interest in the compound. This finding provided support for Szent-Gyorgyi's contention that methylglyoxal may be acting as a natural cell division inhibitor. The reported isolation of methylglyoxal from a structural protein extract of beef liver by Fodor et al. [12] in 1978 provided more direct evidence for its possible semiconductor effect. In addition, Alexeev et al. [21] reported that tumor homogenates do not contain the enzymatic system involved in methylglyoxal formation while muscle homogenates do.

Effects of exogenous methylglyoxal on plant cells have been noted by others on rare occasions [22, 23]. The findings reported here provide evidence that methylglyoxal may be acting as a natural cell division inhibitor in plants. Finding methylglyoxal in an insoluble needle extract allows it to be operating in the proposed semiconductor inducing mode. The resultant charge-transfer reaction between methylglyoxal and structural protein could promote a semisolid cellular interior in needles. Since cell division involves a complete rearrangement of the cellular interior, it is possible only in a semiliquid state which the lack of methylglyoxal in needle callus could allow leading to the observed proliferation. This distribution of methylglyoxal is further supported by our observations on the enzymes of methylglyoxal metabolism in these two tissues and cellular responses to methylglyoxal as described in the accompanying paper [24]. In that light, the recovery of methylglyoxal from an insoluble fraction of only the nonproliferative tissue in the present investigation rules strongly in favor of an in vivo existence in this case.

ACKNOWLEDGMENT

We thank Dr. L.L. Winton and Mrs. Shirley Verhagen for their assistance in tissue culture aspects of this research which was supported by the pulp and paper industry through The Institute of Paper Chemistry. Portions of this work were used by one of the authors (M.M.S.) as partial fulfillment of the requirements for the Ph.D. degree at The Institute of Paper Chemistry.

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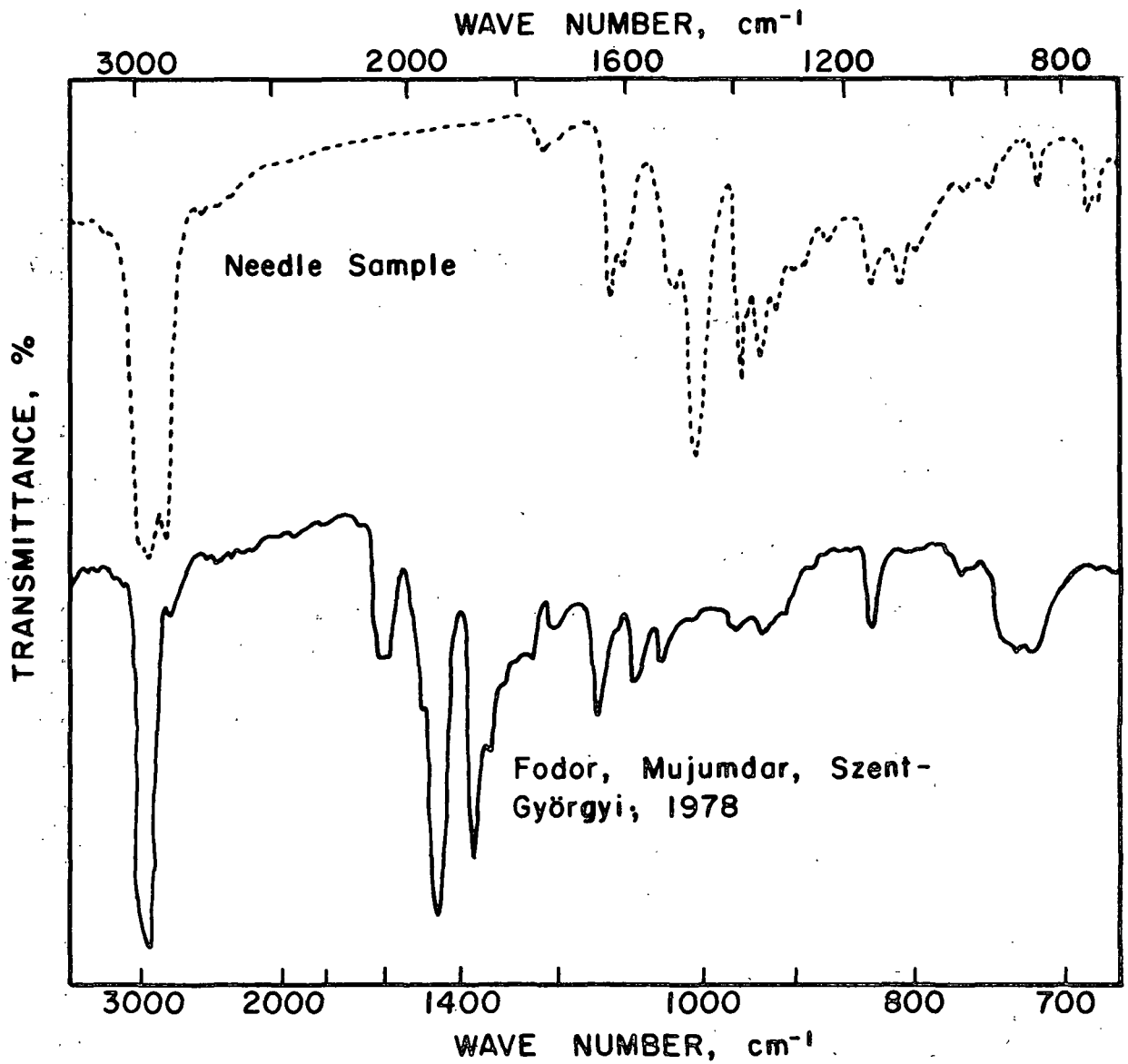

Fig. 1 IR spectra of methylglyoxal osazone from Douglas-fir needles and from commercial methylglyoxal by Fodor et al. (12).

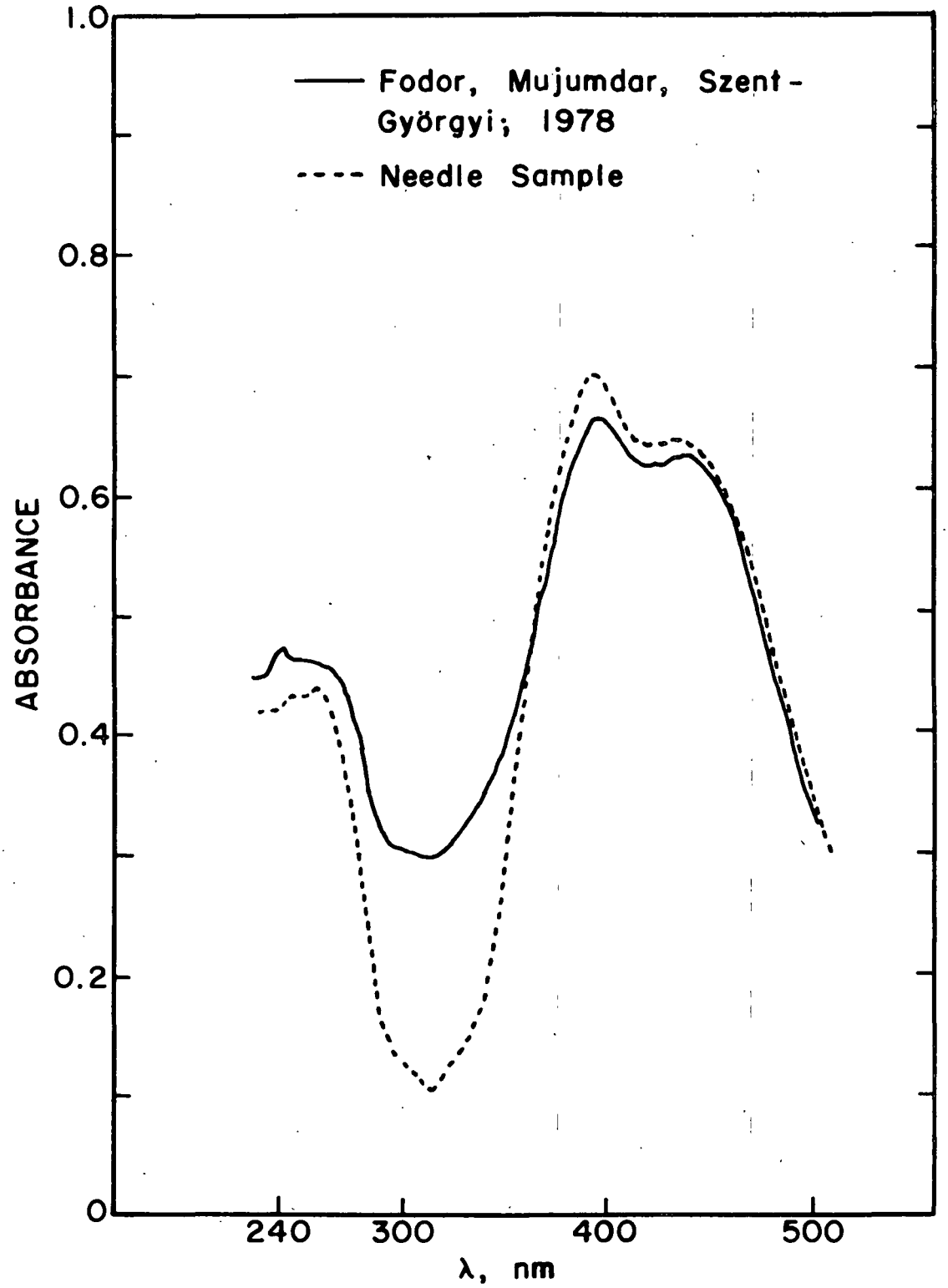

Fig. 2 UV-VIS spectra of Douglas-fir needle sample and of known methylglyoxal osazone by Fodor et al. (12) in chloroform.

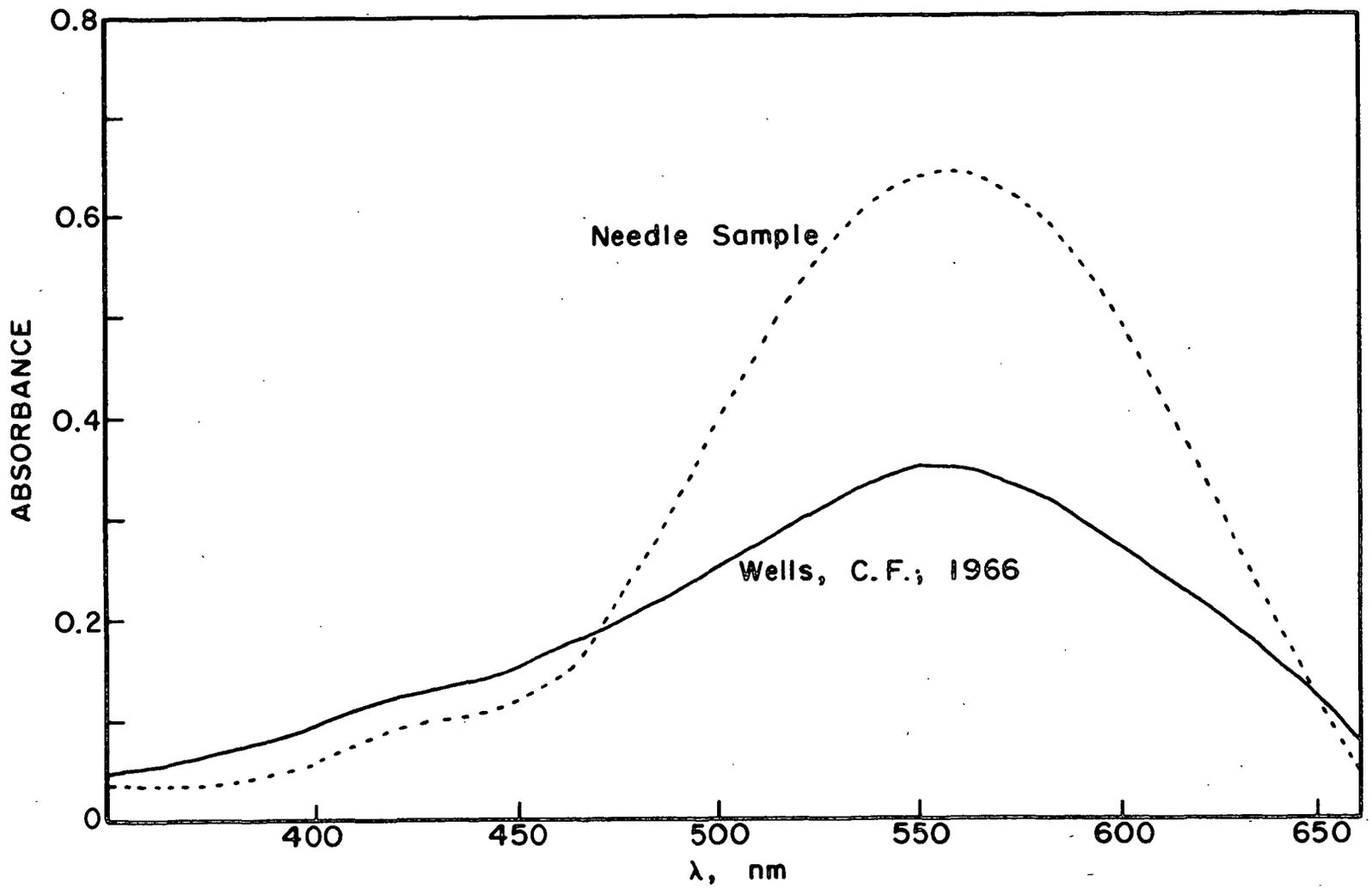

Fig. 3. Visible spectra in methanolic KOH of Douglas-fir needle sample and known methylglyoxal osazone by Wells (14).